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14. ABSTRACT

The present study examined the neuroprotective effectiveness of dantrolene in the prevention of soman-induced SRBD. (Dantrolene is FDA approved for the treatment of malignant hyperthermia.) The decision to consider this compound is based on its well-known mode of action in preventing the release of calcium from intracellular stores. In addition, we assessed possible synergistic overlap in the neuroprotective effectiveness of combined dantrolene and diazepam co-treatment. The combined neuroprotective effectiveness of diazepam+dantrolene is compared with that of diazepam+HU-211 (dexanabinol). The present results provide strong evidence that, by blocking the release of calcium from intracellular stores, dantrolene synergistically augmented the neuroprotection produced by diazepam alone.

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ABSTRACT

It is well known that termination of seizures using anticonvulsant drug therapy is the most effective means of preventing soman-induced seizure-related brain damage (SRBD), i.e., neuronal necrosis resulting from glutamate-induced excitotoxicity. Soman-induced seizures, however, become refractive to anticonvulsant therapy within 40 minutes after onset and development of status epilepticus. Even in cases where seizure termination is successful, excitotoxicity that has already been initiated by seizures of sufficient duration (i.e., 15 minutes or longer) will continue to develop over the ensuing several hours in accordance with well-defined pathways leading to neuronal necrosis or apoptosis. Our laboratory has previously demonstrated proof of concept that a classical neuroprotective approach (i.e., the prevention of delayed calcium overload [Randall and Thayer, 1992]) can interrupt the excitotoxic sequence initiated by seizures and block the development of soman-induced SRBD; this has been accomplished despite the continued presence of status epilepticus. The present study examined the neuroprotective effectiveness of dantrolene in the prevention of soman-induced SRBD. (Dantrolene is FDA approved for the treatment of malignant hyperthermia.) The decision to consider this compound is based on its well-known mode of action in preventing the release of calcium from intracellular stores. In addition, we assessed possible synergistic overlap in the neuroprotective effectiveness of combined dantrolene and diazepam co-treatment. The combined neuroprotective effectiveness of diazepam+dantrolene is compared with that of diazepam+HU-211 (dexanabinol). Male Sprague-Dawley rats were challenged with 180 µg/kg soman (i.e., 1.6 LD_{50}, s.c.). Experimental animals were subsequently given four i.v. injections of 2.5 mg/kg dantrolene at 5 min, 1 hr, 4 hr and 8 hr after seizure onset (i.e., 10 mg/kg dantrolene total, in a solution containing 165 mg/kg mannitol). Alternatively, rats were administered diazepam (20 mg/kg, i.m.) at 40 min after seizure onset; this was immediately followed by the first of four i.v. dantrolene injections (i.e., above solution at 40 min, 100 min, 4 hr and 8 hr after onset). A third experimental group received 20 mg/kg diazepam followed by 25 mg/kg HU-211 at 40 minutes after seizure onset. All rats also received i.p. HI-6 (125 mg/kg, 30 min prior to soman) and i.m. atropine methylnitrate (2 mg/kg, < 1 min following soman) to protect against the peripheral effects of soman. Behavioral signs of soman intoxication and body temperatures were monitored during the first 10 hr after soman administration. ECoG recordings were obtained between 1.5 hour prior to and 6 hours following soman injections. Rats were euthanized 29 hr after soman administration. H&E-stained sections were examined for classical histopathology. The present results provide strong evidence that, by blocking the release of calcium from intracellular stores, dantrolene synergistically augmented the neuroprotection produced by diazepam alone. Despite 40 min of unabated seizures, rats showed complete elimination of convulsive behavior, greatly attenuated ECoG amplitudes (although seizures remained) and recovery. In addition to the prevention of delayed calcium overload by dantrolene, the free radical scavenging properties of mannitol may have provided an extra measure of neuroprotection; since the injected solution was isotonic, it is unlikely that mannitol protected against edema. Unexpectedly, an additional factor almost certainly contributed to the neuroprotection observed in the group receiving diazepam+dantrolene: rats belonging to this group exhibited body temperatures well below the defined

neuroprotective range of 33°C; i.e., body temperatures remained between 30-32°C for more than 10 hr following the diazepam+dantrolene initial treatment. Although it is well known that benzodiazepines (e.g., diazepam) produce hypothermia, it was not clear that a single administration, alone or in conjunction with dantrolene, could produce lasting neuroprotective hypothermia in a soman model. It is, therefore, concluded that combined diazepam+dantrolene therapy offers promise as a viable neuroprotection regimen.

INTRODUCTION

Protection against brain damage resulting from nerve agent exposure is of significant military concern. The current regimen of antidotal therapy most effectively addresses the acute life threatening consequences of exposure. However, many soldiers surviving the initial life threatening effects of nerve agent intoxication are likely to develop seizures. Anticonvulsants such as diazepam can arrest soman-induced seizures when administered shortly following seizure onset, but their effectiveness diminishes when treatment is delayed for 40 minutes or more (e.g., Lipp, 1972, 1973; Shih, 1990; Shih *et al.*, 1991; Capacio and Shih, 1991; Philippens *et al.*, 1992; Sparenborg *et al.*, 1993; McDonough and Shih, 1993; Harris *et al.*, 1994; Shih *et al.*, 1999; Lallement *et al.*, 2000; McDonough *et al.*, 2000). Unless seizures are terminated, the currently fielded therapy does not afford complete protection against brain damage. Furthermore, it is very likely that there will be unconscious nerve agent exposure victims who suffer nonconvulsive seizures that would not be clinically apparent, because these seizures are not associated with the usual behavioral manifestations seen with typical seizures. These victims may not receive any anticonvulsant treatment. If left untreated, or if they cannot be arrested, nerve agent-induced seizures progress to status epilepticus and lead to extensive brain damage. Therefore, there is a clear need for a neuroprotective compound that is capable of preventing brain damage even after seizures have progressed to status epilepticus and cannot be terminated. Such a compound would greatly increase the window of opportunity for the prevention of brain damage resulting from nerve agent-induced seizures and would augment the beneficial effects of currently fielded anticonvulsants.

Soman (O-1,2,2-trimethylpropylmethyl-phosphonofluoridate) is an organophosphorous nerve agent that produces status epilepticus and seizure-related brain damage (SRBD) (Petras, 1981; Lemercier *et al.*, 1983; McLeod *et al.*, 1984; McDonough *et al.*, 1987). Seizure induction results from soman's ability to irreversibly inhibit acetylcholinesterase (AChE), causing an elevation in acetylcholine concentration in the brain (reviewed in Solberg and Belkin, 1997). Once initiated by elevated acetylcholine concentrations in susceptible brain regions, seizures are maintained by excess glutaminergic synaptic transmission (Olney *et al.*, 1983; Sparenborg *et al.*, 1992; Solberg and Belkin, 1997). There is considerable evidence that glutamate receptors mediate soman-induced SRBD. For example, it has been reported that soman-induced status epilepticus and the resulting brain damage can be alleviated by administration of N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonists such as dizocilpine (MK-801) and NBQX (2,3-dihydroxy-6-nitro-7-sulphamoylbenzoquinoxaline), respectively (Braitman and Sparenborg, 1989; Shih, 1990; Sparenborg *et al.*, 1992; Lallement *et al.*, 1993; McDonough and Shih, 1993; Lallement *et al.*, 1994; Solberg and Belkin, 1997). Seizure-related brain damage resulting from soman intoxication is bilaterally symmetrical and most severe in the piriform and entorhinal cortices, amygdala, hippocampus and thalamus (Petras, 1981; Lemercier *et al.*, 1983; McLeod *et al.*, 1984; Pazdernik *et al.*, 1985; Carpentier *et al.*, 1990; Petras, 1994; Ballough *et al.*, 1995, 1998).

Several studies have demonstrated the neuroprotective efficacy of blocking glutamate-mediated excitotoxicity following soman-induced seizures and status

epilepticus (e.g., Braitman and Sparenborg, 1989; Shih, 1990; Sparenborg et al., 1992; Ballough 1998, Lallement et al., 1997; Filbert et al., 1999; Carpentier[a] et al., 2001; Carpentier[b] et al., 2001; De Groot et al., 2001). Recent evidence indicates that a large proportion of the neurotoxic calcium that is elevated intracellularly following NMDA receptor activation originates from an intracellular pool, and its release can be blocked by the skeletal muscle relaxant dantrolene (e.g., Mody and MacDonald, 1995; Yoon et al., 1996; Wei and Perry, 1996; Neibauer and Gruenthal, 1999; Pelletier et al., 1999; Yu et al., 1999; Wei et al., 2000; Nakayama et al., 2002). This approach may offer a novel and alternative therapy against NMDA receptor-mediated excitotoxicity without the unwanted side effects produced by NMDA receptor antagonists themselves (i.e., neurotoxicity in posterior cingulate and retrosplenial cortices). Dantrolene is FDA approved for treatment of malignant hyperthermia. The present study was undertaken to investigate the possibility that dantrolene, alone or in conjunction with anticonvulsant therapy (i.e., diazepam), may be neuroprotective following soman-induced seizures and status epilepticus. The effectiveness of combined diazepam + dantrolene treatment was compared with that of diazepam + HU-211 and diazepam alone.

METHODS

Seventy-one male Sprague-Dawley rats (CRL: CD[SD]-BR; Charles River Labs, Wilmington, MA), weighing between 250-300 g, were used. Animals were housed individually in polycarbonate cages under conditions of constant temperature ($21 \pm 2°C$) and humidity ($50 \pm 10\%$), using at least 10 complete air changes per hour of 100% fresh air, and a 12-hour light-dark cycle (full spectrum lighting cycle with no twilight). Throughout the study, food and water were available *ad libitum*, except during the observation period, which began 1.5 hours prior to and ended 10 hours following soman administration. In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as adopted and promulgated by the National Institutes of Health publication 86-23.

Each rat was anesthetized with sodium pentobarbital (50 mg/kg, diluted in saline to give an intraperitoneal [i.p.] injection volume of 3.3 ml/kg) and positioned in a stereotaxic apparatus (David Koff Instruments, Tujunga, CA); for most subjects, supplemental injections of dilute pentobarbital (i.e., 10 ± 3 mg/kg) were given prior to completion of surgeries, as needed. Prior to incisions, subcutaneous (s.c.) xylocaine was administered for local analgesia. Placement of screw electrodes was performed in accordance with the procedure recommended by Braitman and Sparenborg (1989) for electrocorticographic (ECoG) recordings. Electrodes were connected to a standard small-animal headpiece and secured by dental cement.

On the morning of the fifth or sixth day following surgeries, animals were connected to an ECoG recording system and allowed at least 30 min to acclimate. Baseline ECoG activity and behavior were monitored for at least 15 min. Following baseline recordings, animals were injected (i.p.) with 125 mg/kg of the oxime HI-6. This was followed 30 min later by injection of 180 µg/kg soman (1.6 LD_{50}, s.c.) or sterile saline. Within one min following soman or saline injection, animals were injected intramuscularly (i.m.) with 2 mg/kg atropine methylnitrate (AMN). HI-6 and AMN were

employed to protect against the peripheral effects of soman and to reduce mortality without affecting seizures. Seizure onset, following soman administration, corresponded to the initiation of sustained ECoG amplitudes greater than four times baseline (e.g., McDonough and Shih, 1993). Treatment drugs included diazepam, HU-211 (dexanabinol, Pharmos Ltd., Rehovot, Israel) and dantrolene (two sources: Sigma-Aldrich Co.; Procter & Gamble Pharmaceuticals). Dantrolene injections were prepared by combining "Dantrium" (Procter & Gamble Pharmaceuticals) with purified dantrolene (Sigma-Aldrich Co.); the resultant solution contained 3 mg/ml dantrolene plus 50 mg/ml mannitol. Treatment groups were as follows:

1)	Soman-positive controls,	n = 18
2)	Soman + Dantrolene,	n = 10
3)	Soman + Diazepam,	n = 10
4)	Soman + Diazepam + HU-211,	n = 11
5)	Soman + Diazepam + Dantrolene,	n = 8
6)	Saline + Dantrolene,	n = 2
7)	Saline + HU-211,	n = 2
8)	Saline + Diazepam,	n = 2
9)	Saline + Diazepam + HU-211,	n = 3
10)	Saline + Diazepam + Dantrolene,	n = 3
11)	Untreated controls (sham operated),	n = 2

Note: The above non-soman control groups (6-11) were subsequently collapsed into one group, when it was determined that individual subjects showed no evidence of neuropathology.

In diazepam-treated animals, diazepam was injected 40 minutes after seizure onset (i.e., two i.m. injections of 10 mg/kg each, totaling 20 mg/kg) or 50 minutes following saline injections in controls. In rats that received dantrolene, but not diazepam, the first of four i.v. injections (2.5 mg/kg dantrolene + 41.3 mg/kg mannitol per injection) was administered at 5 minutes following seizure onset. For this group, the remaining three dantrolene injections were administered at 1, 4 and 8 hours following seizure onset. Thus, a total of 10 mg/kg dantrolene + 165 mg/kg mannitol were administered to each of these animals within 8 hours following seizure onset. For the group that received diazepam + dantrolene, rats were i.m. injected with diazepam (as indicated above) 40 minutes following seizure onset; this was immediately followed by the first of four i.v. injections of dantrolene (as described above). For this group, animals received dantrolene injections at 40 minutes (more accurately 42-45 min.), 100 minutes, 2 hours and 4 hours following seizure onset. Rats in the group that received diazepam + HU-211, were i.m. injected with diazepam (as described above). This was immediately followed by i.p. injection of 25 mg/kg HU-211 (the oil Miglyol served as the vehicle). Respective soman-negative controls rats received diazepam (as described above) immediately followed by i.p. HU-211. All rats belonging to the untreated control group received HI-6 and AMN as described above.

In light of the FDA approved use of dantrolene to reduce body temperatures in human cases of malignant hyperthermia, it was of interest to measure body temperatures of rats that received dantrolene alone or in conjunction with diazepam. Body temperatures were obtained from animals belonging to the latter group, as well as to the

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soman positive control and untreated control groups, using rectal probes at 10 hours following soman or saline administration.

For each soman-intoxicated animal, ECoG activities were monitored for a period spanning 1 hour prior to and 6 hours following soman administration. These recordings are currently being analyzed using fast Fourier transform power spectral analysis of the standard frequency bands (i.e., delta [1-3.5 Hz], theta [4-7.5 Hz], alpha [8-12.5 Hz], beta1 [13-20.5 Hz] and beta2 [21-31.5 Hz]).

Twenty-nine ± 1 hours after soman administration, rats were given a lethal injection of pentobarbital anesthesia (130 mg/kg, i.p.) and euthanized. Upon evidence of labored breathing, the animals were transcardially perfused with ice cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were immediately excised and longitudinally divided into left and right hemispheres. Alternate hemispheres (left or right) were stored in 10% neutral buffered formalin, pending paraffin processing. The latter hemispheres were paraffin processed, sectioned at 4 μm and stained with hematoxylin and eosin (H&E). H&E stained brain sections were assessed for classical histopathological damage to the piriform cortex, amygdala, laterodorsal thalamus, hippocampus, and dorsal and lateral cortices. Sections were assessed between bregma - 3.3 ± 0.2 and -4.8 ± 0.2 mm (Paxinos and Watson, 1986). Damage was scored on a scale of 0 to 4, where 0 = no histologic lesion, 1 = minimal damage (1-10% neuronal loss), 2 = mild (11-25% neuronal loss), 3 = moderate (26-45% neuronal loss) and 4 = severe (> 45% neuronal loss).

Contralateral hemispheres were post-fixed by immersion in a second solution of ice-cold 4% paraformaldehyde in 0.1M PB for 4-6 hours. These hemispheres were subsequently sucrose-saturated (30% sucrose in 0.1M PB, for 72 hours) and cryostat sectioned, in the coronal plane, at 40 μm. Serial sections were cryoprotected, in accordance with the procedure of Watson *et al.* (1986), and stored at -20° C. These sections will be immunocytochemically stained for microtubule associated protein-2 (MAP2), pending morphometric image analysis and macroscopic lesion volume determination.

H&E-stained brain sections from all subjects were examined for evidence of neuropathology. Qualitative damage ratings were grouped, according to treatment and brain region, and compared using Kruskal-Wallis and Mann-Whitney nonparametric statistical analyses. In all cases, values for p < 0.05 were considered significant.

RESULTS

All soman-treated rats exhibited sustained seizures and status epilepticus for several hours. Proconvulsive behavioral signs of soman intoxication included repetitive chewing, facial and forepaw clonus, motor stereotypy, and wet-dog shakes. Overt motor convulsions were characterized by rhythmic clonic jerks of both head and forepaws, rearing, salivation and Straub tail. Non-soman control rats showed no evidence of seizures or convulsions. Seizure intensities were reduced (based on visual inspection of ECoG amplitudes) in all animals that received diazepam, but in no cases were seizures arrested. In addition, convulsive behavior was also reduced in these animals. In all rats that received dantrolene, convulsive behavior was virtually eliminated for approximately

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one hour following each dantrolene injection. However, seizure amplitudes appeared to be unaffected. HU-211 did not diminish seizure intensities, nor did it affect convulsive behavior.

Histopathological evaluations of H&E-stained brain sections from rats belonging to the soman-positive control group (i.e., not receiving diazepam, dantrolene or HU-211) revealed severe region-specific brain damage. In these animals, some brain regions exhibited severe lesions, while other regions were virtually unaffected. This damage was bilaterally symmetrical and characterized by widespread tissue necrosis, neuronal loss, chromatolysis, vacuolization, pyknosis and gliosis. The most severe damage was consistently observed in the piriform and entorhinal cortices, dorsal endopiriform nucleus and the laterodorsal thalamic nucleus. Pronounced damage was often seen in the perirhinal cortex, amygdaloid complex (i.e., lateral, basolateral and posteriolateral cortical amygdaloid nuclei), hippocampus (i.e., hippocampal fields CA1 and CA3), midline thalamic nuclei (e.g., mediodorsal, ventromedial), and ventrolateral thalamic nuclei (e.g., ventrolateral and ventroposteriolateral). The pattern of soman-induced seizure-related brain damage (SRBD) seen in the present study is consistent with previous reports (e.g., Petras, 1981; Lemercier et al., 1983; Pazdernik et al., 1985; Carpentier et al., 1990; Ballough et al., 1995, 1998; McDonough et al., 1998). Rats receiving the test drugs showed varying degrees of neuropathology in these same brain regions (see below). Non-soman control rats showed no evidence of neuropathology.

Histopathological damage ratings for H&E-stained brain sections are based on the presence of necrotic neurons and/or the absence of a defined neuronal population. Shrunken neurons are considered the result of artifactual change. Damage to the neuropil is progressively greater as ratings increase from "mild" to "severe," and is characterized by increasingly severe malacia and hyalinization typical of necrosis. For each brain region, respective treatment group means are graphed and presented in Figures 1-3. (Please note that in all references to diazepam, HU-211, and dantrolene-treated groups, soman intoxication is implied.) In Figure 1, it can be seen that a reduction in piriform cortical damage was observed in all groups that received diazepam. No additional protection was produced by co-administration of either HU-211 or dantrolene. Mean piriform cortical neuropathology ratings were 3.78 ± 0.10, 3.20 ± 0.25, 3.18 ± 0.23 and 2.88 ± 0.52 for the soman control, diazepam, diazepam+HU-211 and diazepam+dantrolene groups respectively (for the soman control vs. diazepam groups, p = 0.033). In the amygdala, a reduction in neuropathology was again observed in the diazepam-treated groups (Fig. 1). This was not affected by the addition of HU-211, but did appear to be augmented by the addition of dantrolene (not significant, see below). Mean damage ratings in amygdala for the soman control, diazepam, diazepam + HU-211 and diazepam + dantrolene groups were 3.94 ± 0.06, 3.50 ± 0.17, $3,36 \pm 0.20$ and 2.62 ± 0.46 respectively (for the soman control vs. diazepam groups, p = 0.008; for the diazepam vs. diazepam+dantrolene groups, p = 0.10). The pattern of treatment effects seen in the amygdala seemed to be repeated in the laterodorsal thalamus (Fig. 2). Significant neuroprotection was observed in all groups that received diazepam; however, this was not augmented by co-administration of either HU-211 or dantrolene. Despite the appearance of additional protection by dantrolene co-administration, damage ratings were not significantly lower in the diazepam+dantrolene group compared with the diazepam only group (see below). Mean laterodorsal thalamic damage ratings for the soman control,

diazepam, diazepam + HU-211 and diazepam + dantrolene groups were 3.89 ± 0.08, 3.20 ± 0.25, 3.00 ± 0.14 and 2.50 ± 0.46 respectively (for the soman control vs. diazepam groups, p = 0.006; for the diazepam vs. diazepam+dantrolene groups, p = 0.24). In the dorsal cortical areas, diazepam significantly reduced neuropathology ratings (Fig 3). Neuroprotection was not increased by HU-211 co-administration, but this time it was significantly augmented by dantrolene co-administration (see below). Mean dorsal cortices ratings for the soman control, diazepam, diazepam + HU-211 and diazepam + dantrolene groups were 3.06 ± 0.19, 2.10 ± 0.28, 2.36 ± 0.20 and 1.13 ± 0.30 respectively (for the soman control vs. diazepam groups, p = 0.013; for the diazepam vs. diazepam+dantrolene groups, p = 0.025). A similar pattern of treatment effects was observed in the lateral cortices (Fig. 3). Diazepam significantly reduced damage ratings in all three groups. HU-211 co-administration produced no additional neuroprotection, but dantrolene co-administration did further lower damage ratings (see below). Mean lateral cortices ratings for the soman control, diazepam, diazepam + HU-211 and diazepam + dantrolene groups were 3.33 ± 0.18, 2.50 ± 0.22, 2.64 ± 0.20 and 1.50 ± 0.33 respectively (for the soman control vs. diazepam groups, p = 0.012; for the diazepam vs. diazepam+dantrolene groups, p = 0.027). In the hippocampus, the pattern of treatment effects was somewhat different from that seen in the other five brain regions described above (Fig. 2). While diazepam did significantly lower damage ratings, there was no tendency of dantrolene co-administration to further reduce hippocampal damage. As with the other brain regions, HU-211 co-administration produced no additional neuroprotection. Mean hippocampal damage ratings for the soman control, diazepam, diazepam + HU-211 and diazepam + dantrolene groups were 3.39 ± 0.18, 2.40 ± 0.34, 2.91 ± 0.32 and 2.75 ± 0.56 respectively (for the soman control vs. diazepam groups, p = 0.018). Dantrolene alone (i.e., without diazepam) produced no discernible neuroprotection in any of the brain regions examined.

In most positive control animals, soman produced a reduction in body temperature compared with untreated control animals (mean temperatures were 37.8 ± 0.18 °C and 35.4 ± 0.97 °C respectively; Fig. 4), but this difference was not significant. A significant reduction in body temperature was observed between the soman + dantrolene group compared with the untreated control group (mean body temperature for the former group was 35.2 ± 0.81 °C, p= 0.039). However, no difference was observed between the soman-positive control and the soman+dantrolene groups (means listed above). A further drop in body temperature was seen in the soman + diazepam + dantrolene group compared with the soman-positive controls and also between the former group and animals receiving soman + dantrolene (for the soman + diazepam + dantrolene group, mean body temperature was 31.9 ± 0.78, p=0.37).

DISCUSSION

The present findings demonstrate significant neuroprotection, in all brain regions, by 20 mg/kg diazepam administered 40 minutes following seizure induction by soman. This effect was produced despite the continued presence of seizures and status epilepticus. Even though diazepam did not arrest seizures in this paradigm, it did attenuate ECoG amplitudes, indicating reduced seizure intensities. In addition, a marked reduction in convulsive behavior was also observed in soman-intoxicated animals that

received diazepam. The above effects are consistent with previous reports that diazepam can attenuate soman-induced seizures and convulsions, in rodents and primates, when given shortly following seizure induction (e.g., Lipp, 1972, 1973; Shih, 1990; Shih et al., 1991; Capacio and Shih, 1991; Philippens et al., 1992; Sparenborg et al., 1993; McDonough and Shih, 1993; Harris et al., 1994; Shih et al., 1999; Lallement et al., 2000; McDonough et al., 2000). The present observation that 20 mg/kg diazepam did not arrest status epilepticus in any of the soman-treated animals is also consistent with the above reports which further indicate that diazepam has limited anticonvulsant effectiveness when treatment is delayed for 40 minutes.

The present study also demonstrated that co-administration of 20 mg/kg diazepam + 25 mg/kg HU-211 did not produce additional neuroprotection, in any of the brain regions examined, beyond that produced by diazepam alone. In light of previous evidence that 25 mg/kg HU-211, administered 40 minutes following onset of soman-induced seizures, produces some measure of neuroprotection (Filbert et al., 1999), it is possible that protection produced by diazepam--in the present study--overshadowed the protective effects of HU-211.

The present results indicate that dantrolene administration (i.e., 10 mg/kg dantrolene + 165 mg/kg mannitol administered over an 8-hour period and beginning 5 minutes after seizure onset) produced no evidence of neuroprotection. In a previous study that assessed neuroprotective efficacy of several candidate drugs, it was found that dantrolene alone was capable of producing neuroprotection (Ballough, 1998); however, the dosage of dantrolene employed was ten times greater than that used in the present study. In light of the considerably lower concentration of dantrolene used in this study, and of the fact that dantrolene did not arrest or attenuate soman-induced seizures, it is not surprising that dantrolene injections alone failed to prevent brain damage.

In contrast, co-administration of 20 mg/kg diazepam + 10 mg/kg dantrolene + 165 mg/kg mannitol (i.e., diazepam injected 40 minutes following seizure onset and dantrolene administered over an 8-hour period and beginning 42-45 minutes after onset) had a synergistic neuroprotective effect in the dorsal and lateral cortices, compared with diazepam alone. Although there was not a significant difference between damage ratings of the diazepam alone vs. diazepam + dantrolene groups in the other brain regions, individually (i.e., the piriform cortex, amydala and laterodorsal thalamus), when damage ratings for these groups were combined, and comparisons performed irrespective of brain region, dantrolene co-treatment produced neuroprotection. Therefore, diazepam + dantrolene co-administration produced neuroprotection compared with diazepam alone (in individual or combined brain region comparisons) in all brain regions except the hippocampus. Why this general trend of neuroprotection was not observed in the hippocampus is unclear, particularly in light of several reports indicating that dantrolene reduces hippocampal neuronal injury in several models of glutamate excitotoxicity, including that produced by seizures and status epilepticus (e.g., Niebauer and Gruenthal, 1999; Pelletier et al., 1999).

It is unlikely that mannitol contributed to the neuroprotection observed in the present study. With each administration of 10 mg/kg dantrolene, mannitol was co-administered at 165 mg/kg; the total osmolarity of the injected solution was approximately 288 mosm, which is relatively isotonic. In studies showing reduced edema and neuroprotection by mannitol, dosages typically range between 1.0 - 1.5 g/kg

and produce a hypertonic bolus (e.g., Filbert *et al.*, 1993; Ueno *et al.*, 1994; Berger *et al.*, 1994; Bareyre *et al.*, 1997; Korenkov *et al.*, 2000). However, mannitol has been reported to possess free radical scavenging properties (e.g., Willis *et al.*, 1994; Jiang *et al.*, 2001; Larsen *et al.*, 2002) which may have provided an extra measure of neuroprotection. Interestingly, it was observed that body temperatures in the soman + diazepam + dantrolene group were lowered below 33 °C (i.e., 31.9 ± 0.78 °C) for more than 10 hours. This temperature has been reported to be the threshold below which significant neuroprotective hypothermia occurs in several models of brain damage (e.g., Matsushita *et al.*, 2001; Taylor *et al.*, 2002; Kollmar *et al.*, 2002). Mean body temperatures in the soman-positive control and soman + dantrolene groups were considerably above this threshold. Unfortunately, the decision to measure body temperatures came as an afterthought during the final stage of experimentation, and too few soman + diazepam animals remained for comparison. Therefore, it is uncertain whether neuroprotective hypothermia was the result of the diazepam + dantrolene combination or diazepam alone. It is well documented that benzodiazepines such as diazepam produce hypothermia (e.g., Mailliet *et al.*, 2001; Elliot and White, 2001). In fact, diazepam has been reported to produce hypothermic neuroprotection in other models of brain injury (e.g., Schwartz *et al.*, 1995; Schwartz *et al.*, 1998; Dowden *et al.*, 1999). In addition, it is worth noting that while most animals belonging to the soman-positive control group exhibited reduced body temperatures compared with soman-negative controls (respective medians were 34.7 °C and 37.9 °C ten hours following soman administration), two out of the seven soman-positive control rats that were monitored had body temperatures of 38.6 °C and 39.1 °C. It is tempting to speculate that soman may have precipitated a cytokine-mediated inflammatory reaction in these two animals that was yet to manifest or not induced in the other five rats.

The present study provides strong evidence that, by blocking the release of calcium from intracellular stores, dantrolene synergistically augmented the neuroprotection produced by diazepam alone against brain damage resulting from soman-induced seizures. Moreover, this was accomplished using the dantrolene dosage of 10 mg/kg, which is FDA approved for treatment of malignant hyperthermia. We also provide evidence that the above regimen produces mild hypothermia in the neuroprotective range. In light of the latter findings, it is suggested that future studies using benzodiazepines to mitigate soman-induced seizures should consider the potential benefit of selecting a dosage regimen that produces neuroprotective hypothermia (i.e., body temperatures between 30-33 °C) in addition to reducing the usual ECoG markers. Finally, there is a possibility that the free radical scavenging properties of mannitol provided an extra measure of protection. Future studies are necessary to determine whether higher dosages of dantrolene--in combination with anticonvulsant therapy--can provide more complete neuroprotection against brain damage resulting from soman-induced seizures.
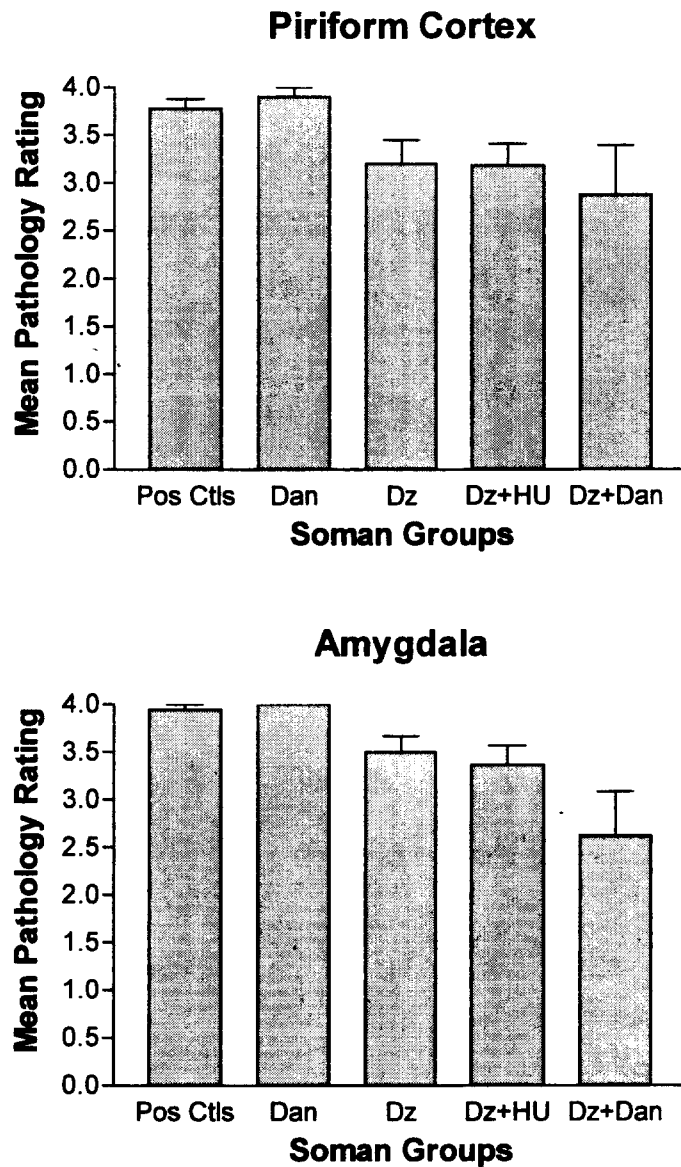
Figure 1

Bar Graphs Depicting Mean Histopathology Damage Ratings ± SEM in the Piriform Cortex and Amygdala. Damage was scored on a scale of 0 to 4, where 0 = no histologic lesion, 1 = minimal damage (1-10% neuronal loss), 2 = mild (11-25% neuronal loss), 3 = moderate (26-45% neuronal loss) and 4 = severe (> 45% neuronal loss). Soman groups include soman-positive controls (Pos Ctls), soman + dantrolene (Dan), soman + diazepam (Dz), soman + diazepam + HU-211 (Dz+HU) and soman + diazepam +dantrolene (Dz+Dan).
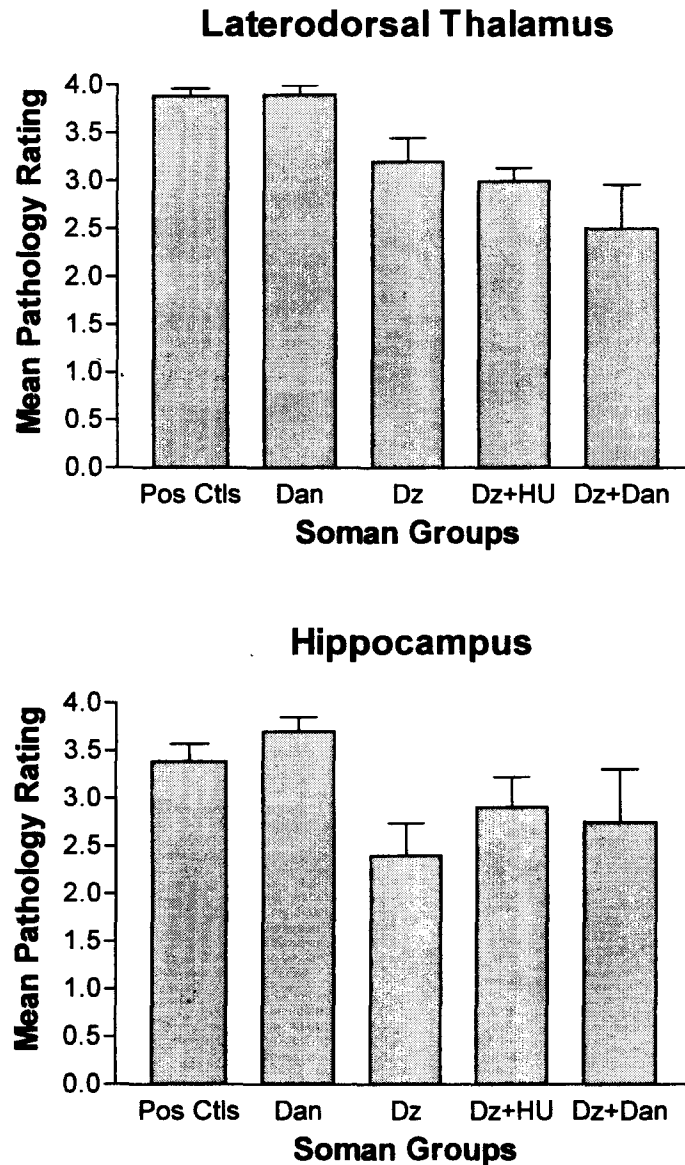
Laterodorsal Thalamus

Hippocampus

Figure 2

Bar Graphs Depicting Mean Histopathology Damage Ratings ± SEM in the Laterodorsal Thalamus and Hippocampus. Damage was scored on a scale of 0 to 4, where 0 = no histologic lesion, 1 = minimal damage (1-10% neuronal loss), 2 = mild (11-25% neuronal loss), 3 = moderate (26-45% neuronal loss) and 4 = severe (> 45% neuronal loss). Soman groups include soman-positive controls (Pos Ctls), soman + dantrolene (Dan), soman + diazepam (Dz), soman + diazepam + HU-211 (Dz+HU) and soman + diazepam +dantrolene (Dz+Dan).
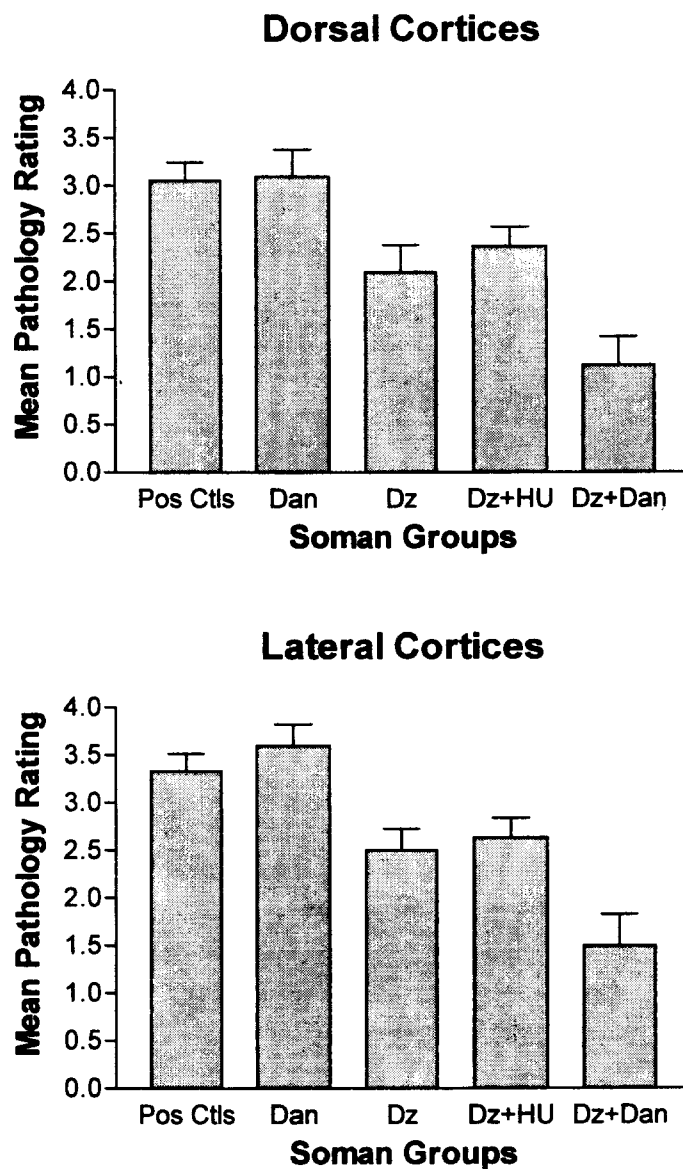
10

Dorsal Cortices

Lateral Cortices

Figure 3

<u>Bar Graphs Depicting Mean Histopathology Damage Ratings ± SEM in the</u>
<u>Dorsal and Lateral Cortices.</u> Damage was scored on a scale of 0 to 4, where 0 =
no histologic lesion, 1 = minimal damage (1-10% neuronal loss), 2 = mild (11-
25% neuronal loss), 3 = moderate (26-45% neuronal loss) and 4 = severe (> 45%
neuronal loss). Soman groups include soman-positive controls (Pos Ctls), soman
+ dantrolene (Dan), soman + diazepam (Dz), soman + diazepam + HU-211
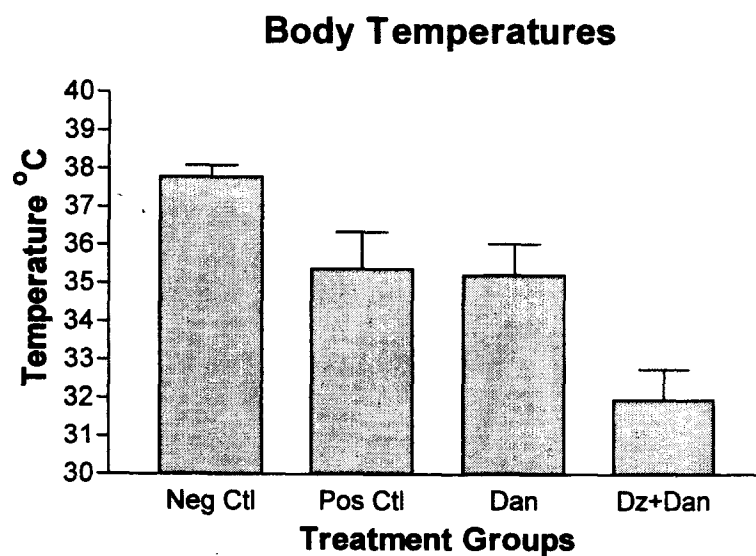(Dz+HU) and soman + diazepam +dantrolene (Dz+Dan).

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Body Temperatures

Figure 4

Bar Graphs Depicting Mean Body (Rectal) Temperatures ± SEM at 10 Hours
Following Soman Administration. Treatment groups include soman-negative
controls (Neg Ctl), soman-positive controls (Pos Ctls), soman + dantrolene (Dan)
and soman + diazepam +dantrolene (Dz+Dan).

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